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A FUNCTIONAL STUDY OF A VARIANT *DOPA DECARBOXYLASE* GENE IN *DROSOPHILA MELANOGASTER*, USING DNA-MEDIATED GERM LINE TRANSFORMATION

BY

ZHI-QING CHEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1986

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Abstract

The expression of the *Drosophila melanogaster* gene encoding dopa decarboxylase *(Ddc)* is temporally controlled. The variant strain Ddc^{+4} shows an altered pattern of *Ddc* gene expression. An examination of the DNA sequences which might control the expression of the gene was undertaken by reintroducing cloned *Ddc* genes from the variant and a control strain into *Drosophila* via P element mediated germ line transformation.

Two P element transposons were constructed by inserting a fragment containing the *Ddc* gene into a plasmid containing the terminal repeats of the P element: the P[Ddc^{+}] transposon, which contained a 7.6 Kb PstI restriction fragment derived from the cloned *Ddc* gene of the control *Drosophila* strain, and the P[Ddc^{+4}] transposon, which contained the same restriction fragment of the *Ddc* gene from the variant strain. Each transposon was co-injected into early embryos of the Canton-S wild type host along with a "helper" P element vector. The transformants were identified by suppression of the lethal phenotype of a *Ddc* mutant allele, which had been introduced into the host by a series of genetic crosses to replace the original wild type *Ddc* gene.

Several transformants were recovered. One of the transformed lines had only 50% absolute dopa decarboxylase (DDC) specific activity of the wild type strain. Cytological mapping showed the exogenous *Ddc* gene integrated near the tip of the left arm of the second chromosome. Its reduced DDC production may be caused by a positional effect. Despite the generally reduced expression in this line, all the reintegrated *Ddc* alleles revealed temporal patterns of *Ddc* expression

characteristic of the strain from which the transforming DNA originally derived. Thus we conclude that the essential information for the expression of the wild type and the variant *Ddc*$^{+4}$ genes was included on the P[Ddc^{+}] and P[Ddc^{+4}] transposons, respectively.

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Introduction

The *Ddc* gene-enzyme system

In *Drosophila melanogaster*, *Ddc* is a single copy gene which encodes dopa decarboxylase (DDC)(Wright *et al.*, 1976; Hirsh and Davidson, 1981; Gietz, 1984), an enzyme which converts 3,4 dihydroxyphenylalanine (dopa) to dopamine (Clark *et al.*, 1978). *Ddc* is subject to tissue-specific and temporal control throughout development. The majority (>90%) of DDC activity occurs in the epidermis (Lunan and Mitchell, 1969), where dopamine is further catalyzed to *N*-acetyldopamine, a compound involved in the sclerotization (hardening and darkening) of newly deposited cuticle (Dickinson and Sullivan, 1975). In addition, DDC activity appears in the central nervous system (Dewhurst *et al.*, 1972; Wright 1977; Livingston and Tempel, 1983), where dopamine presumably functions as a neurotransmitter. The enzyme is also detectable in previtellogenic ovaries (Wright *et al.*, 1981), and in the larval proventriculus (Wright *et al.*, 1982). The enzyme is probably involved in the hardening of the peritrophic membrane of the proventriculus (Rizki, 1956), but its function in ovaries is still unknown.

Five peaks of DDC activity appear in the epidermis throughout ontogeny when hardening of cuticle is required. These stages during the life cycle of the animal are embryonic hatching, both larval moults, pupariation, and adult eclosion (Kraminsky *et al.*, 1980; Marsh and Wright, 1980). Also, five major peaks of the moulting hormone, 20-OH-ecdysone, occur in the life cycle (Kraminsky *et al.*, 1980). A comparison of the hormone and the enzyme profiles indicates that two

distinct situations prevail. At pupariation the hormone titre and the DDC activity increase concomitantly, while at all other stages there is a significant lag between hormone and enzyme maxima. The first evidence of the induction of DDC activity by 20-OH-ecdysone at pupariation was reported by Karlson and Sekeris (1966) in isolated abdomens of *Calliphora*. The role of the hormone in DDC induction in *Drosophila* was investigated using a temperature-sensitive ecdysoneless mutant, ecd^1 (Kraminsky *et al.*, 1980). A rapid increase of the translatable *Ddc* mRNA as well as DDC activity was induced by exogenous feeding of the hormone to mature larvae maintained at the restrictive temperature. Even in the absence of protein synthesis, considerable *Ddc* mRNA accumulation was detected after administering 20-OH-ecdysone (Clark *et al.*, 1986). This implies that the hormone may have a direct effect on transcription of the *Ddc* gene at this stage. Unlike at pupariation, a falling ecdysteroid titer may be required for *Ddc* expression during embryogenesis (Beall and Hirsh, 1984, Gietz and Hodgetts, 1985) and in the imaginal discs following eversion (Clark *et al.*, 1986). These results suggest that the *Ddc* gene responds to different hormonal cues at different developmental stages.

To date, numerous mutant alleles of the *Ddc* gene have been isolated, including: null alleles which behave as recessive lethals, temperature-sensitive alleles (Wright *et al.*, 1981) and two regulatory variant strains, *RE* and *RS*, with approximately 50% more DDC activity than wild type (Sherald and Wright, 1974; Marsh and Wright, 1986). Also, another variant strain selected from a natural population, Ddc^{+4}, has been characterized (Estelle and Hodgetts, 1984a,b). It is not only tissue specific, but also temporally specific. By comparison

with the Canton-S wild type strain, *Ddc*$^{+4}$ has 50% less *Ddc* mRNA and DDC activity at pupariation, when 20-OH-ecdysone has an immediate and perhaps direct effect on the *Ddc* gene, and has 20-50% more DDC activity at the other stages in the life cycle, when DDC activity appears only after 20-OH-ecdysone levels have decreased to the base line. Its phenotype may reflect an alteration of both putative regulatory mechanisms involved in *Ddc* expression. The stage-specific enzyme overproduction and underproduction in *Ddc*$^{+4}$ have both been mapped adjacent to the structural gene for DDC (Estelle and Hodgetts, 1984a), and several differences at the DNA level were found between *Ddc*$^{+4}$ and the wild type strain. These included two small insertions lying in the 5' flanking region of the structural gene (Estelle and Hodgetts, 1984b).

Studies of several *Drosophila* gene-enzyme systems have indicated that at 5' flanking region of the structural gene located the temporal and tissue specific control elements for gene expression and they are separable (Pirrotta *et al.*, 1985; Levis *et al.*, 1985; Lawson *et al.*, 1985). From these observations, one would speculate that rearrangement(s) within this region might lead to abnormal gene regulation. Thus in the *Ddc* system, we would expect that tests of the functional significance of those polymorphisms discovered in *Ddc*$^{+4}$ (mentioned above) might supply some important information about the various mechanisms of ecdysteroid action at this locus. Such functional tests of particular DNA sequences may be performed in *Drosophila* using P-element mediated transformation, which will be described now.

P-element mediated genetic transformation

In *Drosophila*, the development of P element mediated germ line transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982) provided a powerful tool for studying the locality of gene specific regulatory elements. The transfer of *in vitro* modified genes and gene fragments to the germ line can be accomplished by microinjection. The system was developed from the study of a phenomenon called P-M hybrid dysgenesis.

P-M hybrid dysgenesis describes certain correlated genetic aberrations which include high rates of mutation, chromosomal rearrangements, gonadal sterility and male recombination (normally rare in *Drosophila melanogaster)*. They are restricted almost exclusively to the germ line of the F_1 progeny derived from certain matings (Kidwell *et al.*, 1977). They are observed only when males of P strains are mated to females of M strain. P or M indicates a paternal or maternal contribution to the syndrome and such matings are then called dysgenic hybrids. A subsequent study by Engels (1979) showed that the causative factors for thesetraits existed randomly in the genomes of the P strains, and the factors which mapped to the different chromosomes were independent from each other. Further investigations of mutations of the *white* locus (Rubin *et al.*, 1982; Bingham *et al.*, 1982) revealed a family of cross-homologous DNA sequences, P elements, which had inserted in several dysgenic induced *white* mutations. They were present as 30-50 dispersed copies in P strains and absent in M strains (with one exception). The DNA sequencing data (O'Hare and Rubin, 1983) characterized two types of P elements. The first type includes smaller (0.5-1.6 kb), defective elements, which appear to have arisen from the larger ones by different internal deletions. The other type shares a

conserved 2.9 Kb structure which contains sequences from four major open reading frames (ORFs) and are postulated to encode at least two activities: a transposase, required for their own transposition and for the transposition of the defective elements; and a factor which restricts the transposition of P elements only to M strain derived eggs. The 2.9kb element is flanked by inverted repeats of 31bp, whose integrity is required *in cis* for transposition of the P element. Thus hybrid dysgenesis could be explained as follows: when functional P elements carried by the sperm are introduced into an egg of an M strain, they are mobilized to transpose until a critical number of P elements is reached and then accumulated repressor activity restores P element stability (O'Hare and Rubin, 1983). Recently, Laski *et al.*, (1986) and Rio *et al.*, (1986) demonstrated that in the 2.9 kb autonomous P element mRNA the ORFs are joined by the removal of three introns, and the removal of the third intron is required for the production of an 87,000 dalton transposase. They proposed that this critical intron is only removed in the germ line and that its removal is the sole basis for the germ line restriction of P element transformation.

The feasibility of P element mediated germ line transformation in *D. melanogaster* was first demonstrated directly by Spradling and Rubin (1982). They anticipated that a cloned intact P element, if introduced into the germ cells of a developing M strain embryo, would mimic the condition of the dysgenic hybrid. They found that the 2.9kb element could transpose from the bacterial plasmid (into which it had been cloned) and integrate into the chromosomal DNA of the fly's germ line. In an attempt to find out if the transposase supplied by the

intact element could act on a defective element as well, they repeated the experiment by co-injecting the 2.9kb element (as a helper) and a defective one carrying the wild type rosy *(ry$^+$)* gene (the structural gene for an enzyme, xanthine dehydrogenase, found in the synthetic pathway producing one of the eye pigments) (Rubin and Spradling, 1982). The recipient embryos were from a homozygous *ry* mutant strain. Among the progeny of some of the injected embryos, a fraction were shown to have P[rosy$^+$] transposons stably integrated into their genomes. Furthermore, functioning of the integrated *ry$^+$* gene was indicated by the non-mutant eye colour of the injected host and/or their progeny. Thus it was clearly demonstrated that a defective recombinant P transposon could be mobilized in the presence of a transposase supplied by a non-defective helper.

However, because the helper is an intact element, it could transpose itself into the recipient fly genome along with the desired recombinant P element, and result in unstable transformants equivalent to the F_1 progeny of a dysgenic hybrid. This problem was resolved using an improved helper element such as *pπ 25.7 wc* (usually called "wings-clipped") (Karess and Rubin, 1984). With only a single functional inverted repeat, the wings-clipped P element is rendered immobile while supplying the transposase to mobilize a defective recombinant element.

By comparison with other available assays of gene expression, such as cell-free transcription, transient expression in injected oocytes or transfected tissue culture cells, germ line transformation in *Drosophila* offers a number of advantages. The transduced gene is present intrachromosomally, sometimes in proper copy number in all cells, and those cells are in their normal environment in the

developing animal. The study of the expression of the *white* locus is an excellent example facilitated by the system. Hazelrigg *et al.*, (1984) and Gehring *et al.*, (1984) transduced the *white*$^{+}$ gene with >3kb of its 5' region into various genomic sites. They found that the *white*$^{+}$ gene expression was both quantitatively and developmentally normal. The gene was also dosage compensated and interacted with the *zeste* locus as it had in its original location. Using a series of *white* sequences with different small deletions in the 5' region, more detailed information was gained (Pirrotta *et al.*, 1985; Levis *et al.*, 1985). Different parts of the 1.8 kb of 5' region were essential for the expression of the gene in different tissues and at different developmental stages. The gene containing 1.1 kb of 5' region could not express itself in testes of the transformed animals. By contrast, as little as 0.4 kb of 5' flanking region was enough for *white* gene expression in the compound eyes, Malpighian tubules and ocelli of the transformants. It was also found that the sequence required for dosage compensation was contained between position -216 and the transcription start site. The target of *zeste* gene trans interaction was located between 1.8 - 1.1 kb upstream of the transcription start site.

Other successful transformations using P elements include genes for alcohol dehydrogenase (Goldberg *et al.*, 1983; Dudler and Travers, 1984), chorion proteins (DeCicco and Spradling,1984), heat shock proteins hsp 70 (Lis *et al.*, 1983), hsp 26 (Cohen and Meselson, 1985), and hsp 22 (Hulmark *et al.*, 1986), a yolk protein gene (Garabedian *et al.*, 1985), the *period* locus (Zehring *et al.*, 1984), the homeotic gene *fushi tarazu* (Hiromi *et al.*, 1985) and the *E. coli lacZ* gene (Lis *et al.*, 1983) and the neomycin resistance (neoR) gene (Steller and

Pirrotta, 1985).

The system has also been used to study the expression of the *Ddc* gene (Scholnick *et al.*, 1983; Marsh *et al.*, 1985). A 7.6kb cloned wild type gene including both flanking regions was successfully introduced into the *Drosophila* genome. It was found to function in the proper tissues and at the proper stages during development. DDC enzyme activity of all the single inserts was within 30-35% of that found in wild type Canton-S flies. The combination of various transduced *Ddc* inserts yielded a series of strains with up to 10 copies of the *Ddc* gene (Marsh *et al.*, 1985). The enzyme assay data on these recombinant strains confirmed the previous observation that DDC activity is a quantal function of gene dose (Hodgetts, 1975).

As mentioned earlier, functional tests of the polymorphisms of the Ddc^{+4} variant gene, especially those at the 5' region, might reveal the nature of ecdysteroid action at this locus. My approach in the present study was to reintroduce a cloned Ddc^{+4} gene into *Drosophila melanogaster* using P-mediated germ line transformation. We wanted to address the question of the distance between putative cis-acting regulatory element(s) and the structural gene. Since it had already been shown that a 7.6kb Pst I fragment contained the necessary information for *normal* spatial and temporal expression of the *Ddc* gene (Scholnick *et al.*, 1983; Marsh *et al.*, 1985), it was convenient to start by introducing the same fragment from Ddc^{+4}. As a control, the introduction of the Ddc^{+} wild type allele was also done. The main goal was to determine whether this technique would be sensitive enough to examine the subtle quantitative variations of gene expression seen in Ddc^{+4}. If successful, these preliminary experiments would set the stage for testing more restricted DNA fragments from the variant strain.

Materials and Methods

A. Culture Media

The media used for growing all bacterial strains was Luria broth (LB). This contained per liter: 10 g Bacto-tryptone, 10 g NaCl, and 5 g Bacto-yeast extract. Solid media were made of LB plus 15 g/l Bacto-agar. For selection of ampicillin resistance, 100 mg/l ampicillin was added after the media were autoclaved.

B. Bacterial transformation

Transformation of *E.coli* was done according to Cohen *et al.*, (1973). A saturated culture of *E.coli* strain JM 83 or HB 101 was diluted 1/20 in LB and kept shaking for 1 hour at 37°C. Cells were harvested by centrifugation and washed in 10ml of 50 mM $CaCl_2$. The cells were resuspended in 2 ml of 50 mM $CaCl_2$ and left on ice for at least 1 hour before being used. To 0.2 ml of the competent cells, 2-10 ul of DNA solution was added. The mixture was kept on ice for 30 to 60 minutes, then heat shocked at 42°C for 2 minutes. After addition of 1ml of LB, the mixture was incubated at 37°C for 30 minutes. Appropriate dilutions were plated on LB plus ampicillin plates and incubated at 37°C for 12 to 24 hours.

C. Plasmid DNA preparation

A 10ml volume of LB plus ampicillin medium was innoculated with a single bacterial colony and shaken vigorously at 37°C overnight. This saturated culture was used to further innoculate 500 ml of prewarmed LB. The resulting culture was grown at 37°C with vigorous shaking until an OD_{550} of 0.45 was reached. Then 2.5 ml of a 34 mg/ml chloramphenicol

solution was added and shaking was continued at 37°C for 10-16 hours. The cells were pelleted and washed in 100 ml of ice-cold STE (0.1 M NaCl, 10 mM Tris HCl and 1 mM EDTA, pH 7.8). After being resuspended in 10 ml of STE, they were transferred to a 50 ml Erlenmeyer flask and lysed as described by Holmes and Quigley (1981). Briefly, 20 mg of lysozyme was added. The flask was shaken gently over the open flame of a Bunsen burner until the liquid just started to boil. The flask was then immersed immediately in boiling water for 40 seconds and cooled down in ice-cold water. The viscous lysate was centrifuged at 19,000 rpm for one hour. To every ml of the resulting supernatant, 1g of CsCl was added,and an ethidium bromide solution was added to a final concentration of 0.7 mg/ml. The sample was loaded into a VTi 65 rotor and centrifuged at 54,000 rpm for 12-16 hours. The banded plasmid DNA was collected and the ethidium bromide was removed by n-butanol extraction and the $CsCl_2$ was removed in turn by dialysis against two changes of TE (pH 8.0) for 24 hours.

D. Rapid extraction of plasmid DNA

Rapid extraction of plasmid DNA for analytical purposes was done according to Birnboim and Doly, (1979). A 1 ml volume of a saturated culture was centrifuged for 1 minute in an Eppendorf microfuge. The pellet was resuspended in 100 ul of lysis solution (30 mM Tris HCl, 10 mM EDTA, 1% glucose, 0.2 mg freshly added lysozyme, pH 8.0). After incubation on ice for 30 minutes, 200 ul of 0.2 NaOH, 1% SDS was added and the solution kept on ice for another 5 minutes. Chromosomal DNA and protein were precipitated by addition of 150 ul of 3 M Na acetate (pH 5.6), incubation on ice for 45 minutes, and centrifugation for 5

minutes in a microfuge. The DNA in the supernatant was precipitated by the addition of 95% ethanol and incubation at -70°C for 30 minutes. The pellet was resuspended in 100 ul of 1.0 M Na acetate (pH 6.0). The insoluble precipitates were removed by centrifugation for 2 minutes. The supernatant was reprecipitated in 95% ethanol and washed once with 70% ethanol. The dried pellet was resuspended in 100 ul of TE.

E. Phage DNA preparation

The procedure is mainly adapted from Maniatis *et al.*, (1982). A 500 ml volume of LB plus 10 mM $MgSO_4$ and 0.2% maltose was innoculated with 10 ml of saturated NEM 259 and incubated at 37°C with vigorous shaking until OD_{550} of 0.4 was reached. The cell culture was then infected with sufficient plate lysate (approximately 1.6×10^{11} pfu) and maintained without shaking at 37°C for 20 minutes to allow phage absorption. The infected culture was incubated with shaking until extensive lysis occurred (OD_{550} dropped rapidly). Ten ml of chloroform was added and the culture was shaken at 37°C for further 30 minutes. The liquid lysate was cleared by centrifugation following the addition of DNase and RNase (1 ul/ml each) and NaCl (0.7 M) for about one hour. The phage particles were precipitated by addition of solid polyethylene glycol-6000 (10% w/v) and collected by centrifugation at 11,000 x g for 10 minutes. The pellet was suspended in 10 ml of SM buffer (per liter: 5.8 g NaCl, 2 g $MgSO_4.7\ H_2O$, 50 mM Tris, 0.01% gelatin, pH 7.5) and extracted twice with 1 volume of chloroform. To each ml of the recovered aqueous phase, 0.75 g of CsCl was added. The solution was loaded into a SW 50.1 rotor and centrifuged at 35,000 rpm for 24 hours at 4°C. The phage band was collected into an Eppendorf tube. A 1/10

volume of 2 M Tris HCl, 0.2 M EDTA (pH 8.0) solution and 1 volume of formamide were added. After standing at room temperature for at least 30 minutes, 1 volume of H_2O and 6 volumes of 95% ethanol were added followed by immediate centrifugation. The phage DNA pellet was washed in 70% ethanol and resuspended in TE.

F. Restriction enzyme digestion

All the restriction endonucleases were used as recommended by the manufacturer. Typically 1 ug of DNA was digested in 20 ul volume with 5 units of enzyme at 37°C for one hour for plasmid DNA and at least 3 hours for high molecular weight DNA. Reactions were stopped by incubation of the mixture at 65°C for 10 minutes.

G. DNA ligation

Purified DNA samples were mixed in a 3-5 : 1 molar ratio (insert ends to vector ends) in 20-50 ul of reaction buffer which contained 50 mM Tris HCl, pH 7.8, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM adenosine triphosphate, 2mM spermine, and 50 ug/ml bovine serum albumin. After addition of 0.1 unit of T4 DNA ligase the reaction mixture was incubated at 14°C for 8-16 hours.

H. Gel electrophoresis

DNA preparations were routinely analyzed on horizontal submerged agarose gels. A solution of 0.6-0.8% agarose (Sigma type II, medum EEO) was melted in TAE (40 mM Tris acetate, 2 mM EDTA, pH 8.0) and cast in the electrophoresis apparatus. DNA samples were mixed with 1/10 volume of 0.03% xylene cyanol, 0.03% bromophenol blue and 10% glycerol and

loaded onto the gel. The gel was run in TAE plus 0.5 ug/ml of ethidium bromide at 30 volts for 12-20 hours at room temperature. After electrophoresis, the DNA was visualized with long wavelength UV light and photographed with a Polaroid MP4 camera.

I. DNA recovery from agarose gel

A 0.8% low melting point agarose (Sigma) gel was made the same way as described above and the restriction fragments were separated by electrophoresis on the gel. The desired fragment band was cut out of the gel with a razor blade. The slice was melted and passed through an Elutip-d column (Schleicher and Schull) following the manufacturer's instructions. Recovered DNA was ethanol precipitated and resuspended in TE.

J. Nick translation

Radiolabeled nucleotides were incorporated into DNA by nick translation as described by Davis *et al.*, (1980) with minor modifications. The reaction mixture of 50 ul final volume contained 0.5 ug DNA, 50 mM Tris HCl (pH 7.2), 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 ug/ml bovine serum albumin (BSA), 0.2 mM dATP, dGTP, and dTTP, approximately 100 uCi ^{32}P dCTP, 0.02 ng DNase I and 5 units of DNA polymerase I. The reaction was carried out at 14°C for 2 hours, and stopped by the addition of an equal volume of 200 mM EDTA, 2 mg/ml sonicated salmon sperm DNA, and 0.2% SDS. The reacton mixture was loaded onto a column of Sephadex G-50 equilibrated in TE (pH8.0). The nick translated radioactive DNA was collected by the Spun-column procedure described by Maniatis *et al.*, (1982). The total incorporation

was determined by counting an aliquot of the collection in 5 ml of distilled water in a Beckmen L8 7500 liquid scintillation spectrophotometer.

K. Oligo labelling

For genomic southern blots, radioactive probe was made by oligo labelling as described by Feinberg and Vogelstein, (1983,1984), except for the use of purified DNA. The labelling reaction was started by addition of the following reagents in order: H_2O (to a total volume of 50 ul), 10 ul of the oligo labelling buffer (OLB, see later) 2 ul of 10 mg/ml BSA, 5 ng DNA (heated to 95-100°C for 2 minutes followed by immediate ice bath), 50 uCi of ^{32}P dCTP, 2 units of large fragment of DNA polymerase I. The reaction mixture was incubated at 37°C for 12-16 hours and the reaction was stopped by addition of an equal volume of 200 mM EDTA, 2 mg/ml sonicated salmon sperm DNA, 0.2% SDS. Specific activity of the labeled DNA was determined as described above for nick translation. The OLB was made by addition of a 1 ml volume of 1.25 M Tris HCl (pH 8.0), 0.125 M $MgCl_2$ to 18 ul of 2-mercaptoethanol and 5 ul of dATP, dGTP, dTTP (100 mM each). The resulting solution was then mixed with 2 M Hepes (pH 6.6) and hexadeoxyribonucleotides ($Pd[N]_6$, Pharmacia) (in TE at 90 OD units/ml) in a ratio of 100 : 250 : 150. The buffer was stored at -20°C and used repeatedly for up to three months.

L. Filter hybridization

E.coli cells transformed with an hybrid plasmid DNA pool were screened by filter hybridization according to Maniatis *et al.*, (1982). Cells were transformed and plated on a LB plus ampicillin plate as

described earlier. When individual colonies had grown to approximately 1.0 mm in diameter, a nitrocellulose filter was placed onto the plate for several seconds then peeled off with blunt ended forceps. With colony side up, the filter was placed on a piece of Whatman 3MM paper saturated with 0.5 M NaOH for 2-3 minutes and repeated once. The filter was blotted on a dry paper towel and transferred to another 3MM paper saturated with 1 M Tris HCl (pH 7.4) for 5 minutes and repeated. It was then baked for 2 hours at 80°C in a vacuum oven.

For Southern transfers, DNA fragments were separated on an agarose gel and transferred onto a piece of nitrocellulose or diazotized paper as described by Davis *et al.*, (1980). After electrophoresis the gel was placed in a tray with 250 ml of 0.25 M HCl and shaken gently for 30 minutes with one solution change. This solution was then replaced by an equal volume of 0.5 M NaOH and 1.5 M NaCl, and the gel was agitated for another 30 minutes with one solution change. After being neutralized in 500 ml of 0.5 M Tris HCl (pH 7.5) for 30 minutes, the gel was placed on 2 pieces of damp 3MM paper and covered by a piece of nitrocellulose or diazotized paper, 5 sheets of 3MM paper and a stack of about 6 cm of paper towel with a 1 kg weight on top. All the papers were cut to the gel size. Transfer of DNA was allowed to proceed for 12 hours or longer. The filter was soaked in 6 x SSC (150 mM NaCl, 15 mM trisodium citrate) for 5 minutes and baked in a vacuum oven.

Hybridization of the filters to a ^{32}P-labeled probe was done according to Thomas (1980). Each filter was prehybridized in a 10 ml volume of 4 x SSC, 0.1 g Ficoll, 0.1 g polyvinylpyrrolidone, 0.1 g BSA, 40 mM sodium phosphate (pH 6.5), 50% v/v formamide, 250 ug/ml sonicated and denatured salmon sperm DNA and 1% dextran sulfate for 8-16 hours at

42°C. DNA probe mixed with 1 ml of the above solution was denatured by heating to 95°C for 5 minutes, then added to 4 ml of the solution. Hybridization was continued for 8-12 hours at 42°C. The blots were washed with 3 changes of 2 x SSC, 0.1% SDS at room temperature and 0.1% SDS at 50°C. Each wash took 5-10 minutes. Blots were wrapped in Saran wrap and exposed to Kodak X-ray film using an intensifying screen at -70°C.

Genomic Southern hybridizations were done as described by Klessig and Berry,(1983) and oligo labeled probes were used.

M. *Drosophila melanogaster* stocks

All the fly stocks were routinely maintained on a standard yeast-sucrose medium (Nash and Bell, 1968) at 25°C or as indicated elsewhere. Table 1 gives a complete list of all *D.melanogaster* stocks used and their origin.

N. Microinjection

The protocol used for microinjection was described by Rubin and Spradling (1982) with some minor modifications. Embryos were colleced at half hour intervals on 1.5% agar plates spread with yeast. The embryos were dechorionated by rolling on double stick tape on a slide and lined up at the edge of the tape with their posterior end protruding over the edge. After drying over a desiccating silica gel for about 10 minutes, the embryos were covered with halocarbon oil (series 700) and were ready for injection.

The needles were prepared by pulling siliconized 25 ul capillary pipettes to a sharp point in an electrode puller (Aloe Scientific). The

Table 1.

***Drosophila melanogaster* stock list ¶**

Strains	Designation in text
Canton-S	wild type
Ddc^{+4} pr	variant
Ddc^{ts2} pr	*Ddc^{ts2}*
Ddc^{n7}/CyO †	*n7/CyO*
Ddc^{n7}/CyO; Sb/Tm3, Ser *	balancer stock, *n7/CyO; Sb/Ser*
hk (San Diego)	*hk*

¶ Genotype designations and original references are given as in Lindsley and Grell (1967).

† *Ddc^{n7}* chromosome carries *rdo hk Ddc^{n7} pr*
CyO chromosome carries *In(2LR)O, dp Cy pr cn*

* *Sb* chromosome carries *Sb^{63b}*, and
Tm3 chromosome carries *Tm3, rip^{p} sep bx^{34c} e Ser*, respectively. The stock *Sb/Ser* was obtained from Dr. J. Kennison. This stock was mated to the *Ddc^{n7}/Cyo* flies to establish the *Ddc^{n7}/CyO; Sb/Ser* stock. All the other stocks listed were obtained from Dr. R. B. Hodgetts.

tips of the needles were broken to a diameter of about 1 um under 100 x magnification by forcing it into the double stick tape.

The DNA for injection was ethanol precipitated and resuspended in the injection buffer (5 mM KCl and 0.1 mM sodium phosphate, pH 6.8). The final DNA concentration was 300 ug/ml for the transforming vectors and 100 ug/ml for the helper vector, *px25.7 wings clipped*. (obtained from Dr. Marsh)

The slides to which the host embryos were affixed were placed on the microscope stage and a Leitz micromanipulator was used. The manipulator controls were used only to move the microinjection needle into the focal plane; the stage and the focus controls were used to position the embryos.

The injected embryos were grown at 18°C under oil in a slide storage box with high humidity in it. The hatched first instar larvae were transferred to standard media at 25°C and the emerged adults were individually crossed to appropriate chromosomal balancers to screen the transformants and further investigate the integrated gene expression.

O. In situ hybridization to polytene chromosomes

In situ hybridization to polytene chromosomes was performed according to Pardue and Gall (1975). Larvae were grown at 18°C at low density. Late third instar larvae were collected and salivary glands were dissected out in 45% acetic acid. After being transferred onto a new slide, the glands were covered with a siliconized cover slip and squashed by applying a firm thumb pressure on the cover slip. The cover slip was clipped off after immersion in liquid nitrogen for several seconds. The slide was then submerged in 3 : 1 ethanol : acetic acid to

fix for 3 minutes, dehydrated in 95% ethanol for at least 5 minutes (twice), air dried and stored at 4°C. The slides were heated in 2 x SSC for 30 minutes at 70°C, RNase treated (0.1 mg/ml Sigma RNase A in 2 x SSC for 1 hour at 37°C) and denatured in 0.07 N NaOH for 3 minutes. Each treatment was followed by 3 times 70% and twice 95% ethanol dehydration and air drying. Then onto each slide 20 ul of hybridization mixture were added and a cover slip was applied. The mixture consisted of equal volume of denatured DNA (^3H labelled nick translated *Ddc* probe, 3 x 10^4 CPM/ul, and sonicated salmon sperm DNA, 0.4 mg/ml) and hybridization buffer (in 5 ml, contains 0.2 ml 50 x Denhardt's solution, 1.0 ml 50% dextran sulphate, 0.66 ml 5 N NaCl, 0.1 ml $MgCl_2$ and 0.2 ml o.5 M sodium phosphate buffer, pH 7.0). The slides were then placed in a sealed moist chamber, and submerged in a 65°C water bath for 12 hours. To terminate the hybridization reaction, the cover slips were removed and the slides were washed 3 times at 65°C, and twice at room temperature in a 2 x SSC solution. Finally, the slides were ethanol treated and air dried. The dried slides were coated with autoradiographic emulsion (Kodak NTB 2 liquid emulsion diluted 1 : 1 with distilled water) and exposed in a black slide box for one week at 4°C, then developed for 2 1/2 minutes in Kodak D-19 and fixed for 5 minutes in Kodak fixer. After rinsing through several changes of distilled water (15 minutes), the slides were Giemsa stained and coverslips were added.

P. DNA extraction from small numbers of flies

DNA extraction from small numbers of flies was performed according to Coen *et al.*, (1982) with minor modifications. Fifteen flies were

frozen at -70°C for a few minutes in a 1.5 ml microfuge tube containing 200 ul of 10 mM Tris, 60 mM NaCl, 5% sucrose, 10 mM EDTA, pH 7.5, then gently homogenized in the tube. After adding 200 ul of 1.25% SDS, 0.3 M Tris, 0.1 M EDTA, 5% sucrose, 8% diethyl pyrocarbonate (freshly mixed), pH 9.0, the extract was incubated at 65°C. Then 60 ul of 3 M Na acetate pH 5.6 was added and the mixture kept on ice for 45 minutes. The precipitate was spun down for 5 minutes and the supernatant added to 2 volumes of ethanol. After standing for 2 minutes on ice, the ethanol precipitate was spun down for 10 minutes. The pellet was suspended in 200 ul of freshly prepared 0.2% diethyl pyrocarbonate and left to stand at room temperature for 30 minutes. The insoluble preciptate, appearing after centrifugation, was discarded. Three volumes of ethanol were then added and the mixture left on ice for a further 2 minutes. The DNA was spun down for 10 minutes, washed with 70% ethanol twice, vacuum dried for few a minutes and dissolved in 30 ul of Tris buffer pH 7.5.

Q. DDC enzyme and protein assays

DDC enzyme assays were performed mainly according to McCaman *et al.*, (1972). White prepupae and newly emerged adults (0-2 hours) were collected and immediately frozen in the -70°C freezer. Extracts for enzyme assays were prepared at a concentration of 20 mg live weight per ml in the extraction buffer (0.3 M sucrose, 0.2 mM phenylthiourea and 0.1 M sodium phosphate, pH 7.1) in a glass homogenizer. The extract was centrifuged at 27,000 x g for 10 minutes. The supernatant was used for assays. Each extract was assayed for DDC activity in three duplicates. For each assay 5 ul of the extract was added into a 10 ul volume of reaction solution in an Eppendorf. The solution contained 0.4 mM ^{3}H

L-dopa, 0.5 mM unlabelled L-dopa and 0.1 mM pyridoxyl 5' phosphate in 0.1 M sodium phosphate, pH 7.0. The reaction mixture was incubated at 30°C for half an hour. Then the reaction was stopped by addition of 300 ul of wash buffer (0.05 M sodium phosphate, pH 7.0) and 100 ul of 0.1 M diethylhexylphosphoric acid (in chloroform). Phases were mixed by vortexing and separated by a brief centrifugation. Then the aqueous phase was discarded. The organic phase was washed again with 300 ul buffer, followed by centrifugation. Finally, 50 ul of the organic phase, containing the dopamine formed by the enzyme reaction, was taken for scintillation counting. One unit of specific activity is defined as the amount of enzyme activity required to decarboxylate 1 nmole dopa in 30 minutes per mg total protein. Protein concentrations were determined as described by Spector (1978).

RESULTS

A. Construction of the pZ plasmid

To locate the regulatory element(s) of the variant *Ddc*$^{+4}$ gene, the pZ plasmid was constructed. The cloned recombinant phage, λ15 (Estelle and Hodgetts, 1984b), was used as the source of the *Ddc*$^{+4}$ gene. A 7.6 kb Pst I restriction fragment was chosen for the functional test based on the experiments of Scholnick *et al.*, (1983). This includes all of the *Ddc* transcription unit plus 2.5 kb of 5' and 1 kb of 3' flanking region DNA (see Figure 1). There are about twenty Pst I sites on the phage. However, only one of the Pst I fragments is homologous to a *Ddc* probe (data not shown). We subcloned the fragment corresponding to *Ddc*$^{+4}$ into the pHDlac plasmid, obtained from Dr. Larry Marsh (Figure 2), using a "shotgun" strategy, as described below.

λ 15 was digested with restriction enzyme Pst I and ligated with Pst I treated pHDlac DNA as described in Materials and Methods. The resulting recombinant DNA molecules were used to transform *E.coli* strain HB 101 which was plated on LB plus ampicillin. The transformed colonies were screened by filter colony hybridization. A ^{32}P labeled nick translated 1.9 kb Bam HI fragment was used as the probe (probe 1), which contains the second exon and intron and part of the third exon of the *Ddc* gene (see Figure 1). Among the approximately 1,600 colonies screened four hybridized with the probe. They were plated out again and rescreened. One of them was then chosen for further analysis and from a single well-separated colony, a cell culture was made. The plasmid DNA was isolated and analyzed by restriction digestion and gel electrophoresis. The plasmid was designated as pZ and its construction

Figure 1. Restriction map of the *Ddc* region. The approximate location of the *Ddc* transcription unit is indicated above the restriction site map (Estelle and Hodgetts, 1984b; Hodgetts, personal communication). Intron sequences are represented by dashed lines and exon sequences by cross hatched boxes. The asterisk indicates one of the restriction site polymorphisms between cloned Ddc^{+4} and the Canton-S *Ddc* DNA. This site is missing in Ddc^{+4} DNA. The bold lines beneath the restriction site map represent the probes used in this study.

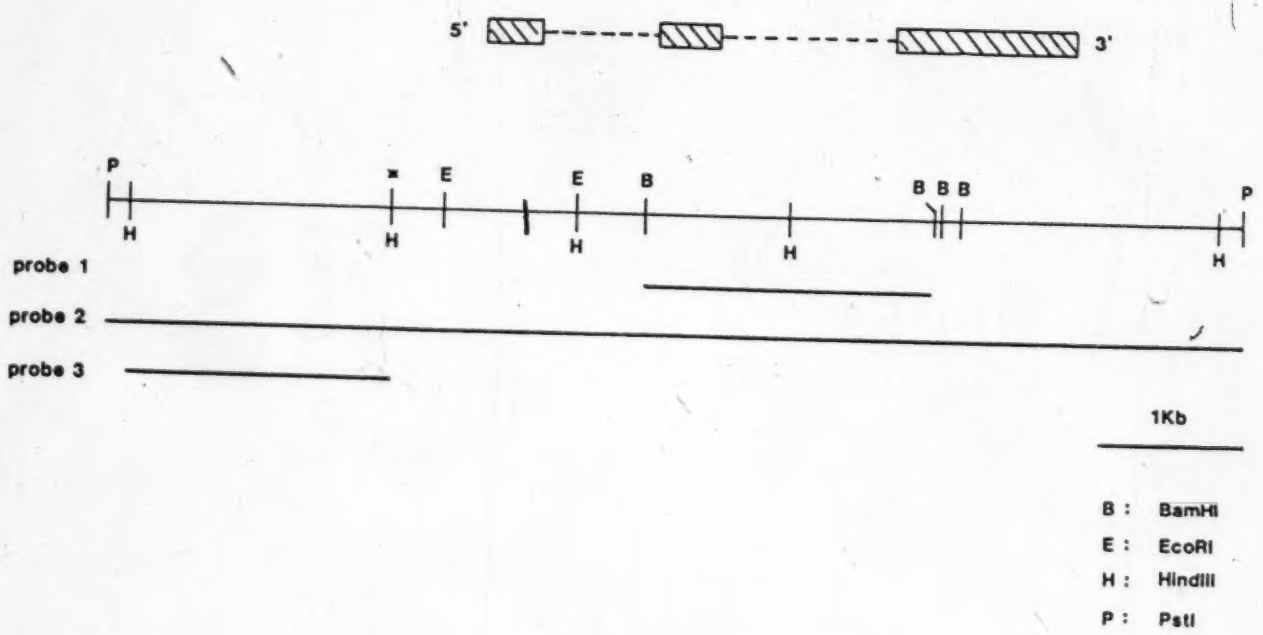
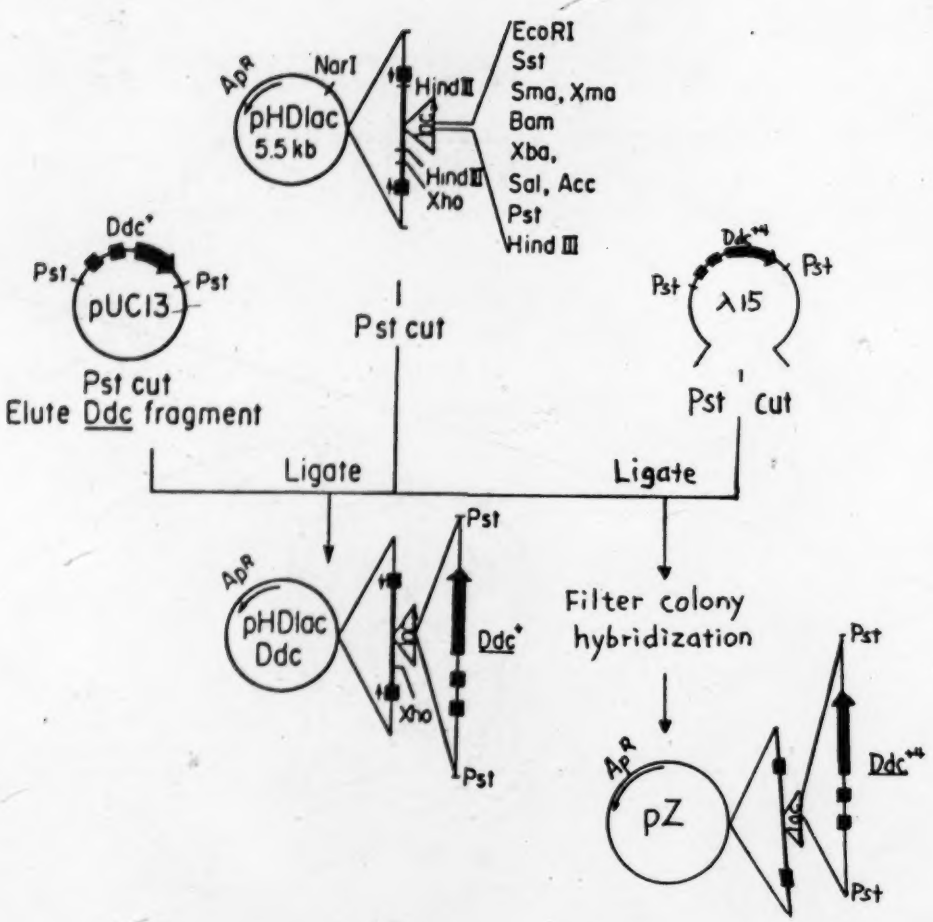

5'
3'
P
H
E
B
B B B
probe 1
probe 2
probe 3
1Kb
B : BamHI
E : EcoRI
H : HindIII
P : PstI

Figure 2. Construction of the hybrid transforming plasmid pZ and p10lac. A recombinant phage λ15 (Estelle and Hodgetts, 1984b) which spanned the entire *Ddc* gene and both flanking PstI sites was used as source of the variant Ddc^{+4} DNA. The phage was digested with PstI and ligated with PstI linearized pHDlac DNA. The resulting recombinant DNA pool was used to transform *E.coli* strain HB101 and plated on LB plus ampicillin. The colony containing the pZ plasmids was screened out with probe 1 (Figure 1) and further examined by restriction analysis.

The p10lac was constructed by ligating a purified 7.6 kb PstI fragment containing the Canton-S wild type derived intact *Ddc* gene to PstI linearized pHDlac DNA. Both pHDlac and p10lac plasmids were obtained from Dr. Larry Marsh.


ApR
NarI
pHDlac
5.5 kb
HindIII
Ddc
HindIII
Xho
EcoRI
Sst
Sma, Xma
Bam
Xba,
Sal, Acc
Pst
Hind III
Ddc+
Pst
pUC13
Pst
Pst cut
Elute Ddc fragment
Pst cut
Ddc+4
Pst
λ15
Pst
Pst cut
Ligate
Ligate
ApR
pHDlac
Ddc
Pst
Ddc+
Xho
Pst
Filter colony
hybridization
ApR
pZ
Pst
Ddc+4
Pst

is summarized in Figure 2.

Another transforming plasmid, p10lac, was also obtained from Dr. Larry Marsh. This plasmid has the 7.6 kb wild type *Ddc*$^{+}$ Pst I fragment inserted in pHDlac vector as depicted in Figure 2.

Figure 3 shows the restriction analysis of pZ and p10lac plasmid DNA. Pst I restriction reveals the similar 7.6 kb insertion fragment for both plasmids. Hind III digestion reveals the known restriction site polymorphism which distinguishes the *Ddc*$^{+4}$ sequences from the Canton-S sequences (Estelle and Hodgetts, 1984b). This site, which is indicated by an asterisk in Figure 1, is missing in the *Ddc*$^{+4}$ DNA. Thus the 5' region of *Ddc* in p10lac was cut into 1.2 and 1.8 kb fragments, by Hind III, whereas this enzyme produced a single 3 kb fragment in pZ. This is just one of the DNA polymorphisms that serves to distinguish the *Ddc* gene derived from Canton-S from the *Ddc*$^{+4}$ variant strain (Estelle and Hodgetts, 1984b).

B. Germ line transformation

The first series of experiments involved the injection of embryos from the *Ddc*^{ts2} strain with the p10lac transforming plasmid. However, the survival rate of the injected embryos was extremely low. The data presented in Table 2 show that out of 964 injected embryos, only 73 hatched and 9 finally developed into adulthood. These data prompted us to examine the "natural" hatch rate of *Ddc*^{ts2}. The embryos were collected, mechanically dechorionated, covered with halocarbon oil and placed in a highly humidified slide store box. These embryos therefore underwent all the manipulations for transformation except injection. Only 45 hatched out of 137 collected embryos (0.33). This implies that

Figure 3. Restriction analysis of the transforming plasmids p10lac and pZ. Approximately 1ug/slot of DNA was restricted with endonuclease and electrophoresed on a 0.8% agarose gel. Slot 1 contains λ DNA restricted with EcoRI which served as molecular weight markers. Slots 2 and 4 contain pZ DNA, and slots 3 and 5 contain p10lac DNA. The DNA in slots 2 and 3 was digested with PstI, in 4 and 5 with HindIII.

The molecular weight of the fastest band in slot 4 was slightly greater (<100 bp) than that in slot 5. The difference seen in these two vector-insert hybrid bands may reflect a polymorphism which had not be detected in the previous study by Estelle and Hodgetts (1984b).

1
2
3
4
5
23.13
9.42
6.68
4.36
2.32
2.03

TABLE 2. Survival rate of microinjected embryos

Recipient Strains	Donor DNA Injected	# Embryos Injected	# Larvae†	# G_0 Adults	# Fertile Adults
Ddc^{ts2}	p10lac	964	73	9	3
Canton-S	p10lac	1444	505	111	72
Canton-S	pZ	427	168	39	27

† First instar

the major cause of lethality amongst the 964 injected *Ddc*^{ts2} host was the manipulations that preceeded injection. The same experiment was done on Canton-S embryos as well. The hatch rate was 0.77 (56/73) under the same conditions. This comparison revealed that *Ddc*^{ts2} was a less suitable transformation host than the Canton-S wild type strain. For this reason, the rest of the microinjections were performed on Canton-S embryos with p101ac or pZ hybrid plasmid DNA. As the data in Table 2 illustrate, the use of the Canton-S strain as a recipient not only yielded more first instar larvae, but also gave more surviving G_0 adults than the *Ddc*^{ts2} host.

C. Identification of the *Ddc*^{ts2} transformants

The nine *Ddc*^{ts2} G_0 flies which survived the injection of p101ac DNA were individually mated to balancer adults of the appropriate sex (see Figure 4a). The resulting G_1 embryos were transferred to 29°C and the vials were scored for the presence or absence of straight winged flies, since all the *Ddc*^{ts2}/*Ddc*^{n7} flies (with straight wings) would die at this restrictive temperature unless there is an integrated *Ddc* wild type gene functioning somewhere in their genome. Therefore, any of the G_0 flies which gave rise to straight winged G_1 progeny were considered as putative transformants (Figure 4a). As shown in Table 3a, T10-c and T10-g did not produce any straight winged progeny and were discarded; T10-d gave the desired offspring and was maintained. To determine the site (or sites) of the *Ddc* insertion, the G_1 adults were selfed. As shown in Table 1, the *Ddc*^{n7} chromosome carries the recessive marker genes *rdo hk* and *pr* linked to the *Ddc* locus. If the exogenous *Ddc* gene

Figure 4. Crossing scheme used to select the putative transformants among *Ddc*^{ts2} derived G_0 adults. *Ddc*^{ts2} G_0 flies were individually mated to the balancer adults of the appropriate sex. The G_1 embryos were then transferred to the restrictive temperature, 29°C. Among the G_1 adults, the *Ddc*^{ts2}/*Ddc*^{n7} flies (straight wings) will not appear unless they are rescued by integrated *Ddc*$^{+}$ gene(s). The G_1 putative transformants were selfed. Among the G_2 progeny: a. if the exogenous *Ddc* gene integrated but into the third chromosome, one would observe *n7* homozygous progeny with *rdo hk pr* phenotype; b. if it integrated into X chromosome, one would get a 1:2 sex ratio; c. if it integrated into the second chromosome, one would never get any *n7/n7* homozygous progeny.

a.

G_0 Ddc^{ts2} / Ddc^{ts2} ; * / + X *n7* / *CyO* ; *Sb* / *Ser*

|

v

G_1 Ddc^{ts2} / *n7* ; *Sb* / *

|

v

G_2 *n7* / *n7* ; *Sb* / *

b.

G_1 X */ X X X */ Y

|

v

G_2 X */ X * : X */ X : X */ Y : X/ Y

1 : 1 : 1 : 1

(die)

c.

G_1 Ddc^{ts2} */Ddc^{n7} X Ddc^{ts2} */Ddc^{n7}

|

v

G_2 Ddc^{ts2} */*n7* : Ddc^{ts2} */Ddc^{ts2} * : *n7*/*n7*

1 : 2 : 1

(die)

* indicates a chromosome carrying a newly acquired *Ddc* gene as a result of P element mediated transformation.

TABLE 3a. G_1 progeny of the injected *Ddc*^{ts2}

G_0 Adults	Sex	Fertility	# G_1 progeny	Phenotypy #Curly wings	#Straight wings
T10-a	F	-	0	-	-
T10-b	F	-	0	-	-
T10-c	F	+	90	90	0
T10-d	M	+	138	132	6†
T10-e	F	-	0	-	-
T10-f	M	-	0	-	-
T10-g	M	+	119	119	0
T10-h	F	-	0	-	-
T10-i	F	-	0	-	-

† Four males and two females.

Table 3b. G_2 progeny of T10-d line

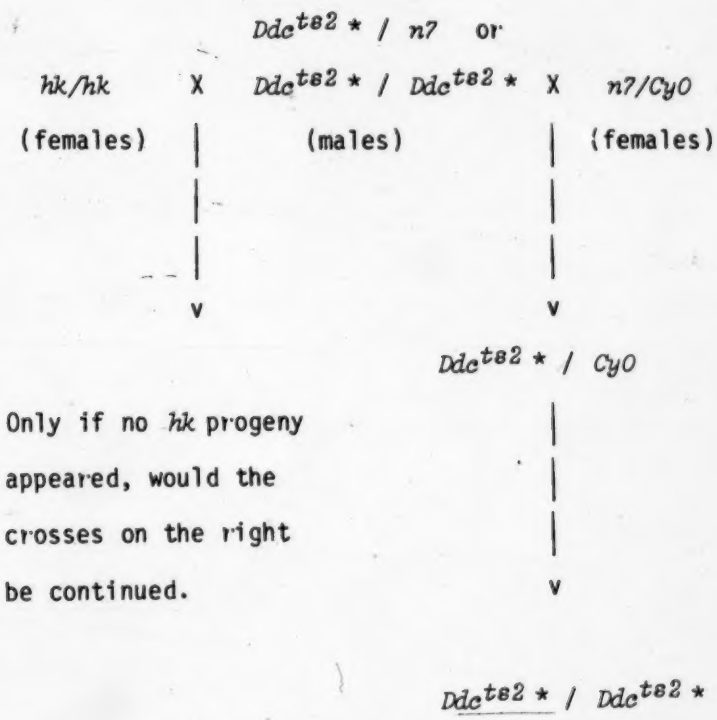
G_0 Adult	# G_2 offspring	*rdo*$^+$ *hk*$^+$ *pr* M	F	*rdo hk pr* M	F
T10-d	86	41	45	0	0

one would expect that *rdo hk pr* flies should appear among the G_2 progeny. If the gene integrated into the X chromosome (Figure 4b), one would expect a 1 : 2 (male to female) sex ratio of the G_2. Since we did not find any *rdo hk pr* flies among the G_2 offspring nor a significant difference between the numbers of the two sexes (Table 3b), we assume that the exogenous *Ddc* gene integrated into the second chromosome (Figure 4c). The G_2 population of T10-d was heterogeneous then, containing both homozygous Ddc^{ts2} */Ddc^{ts2} * and heterozygous Ddc^{ts2} */Ddc^{n7} flies. These can not be distinguished from each other phenotypically since both of them have purple eyes as the only mutant phenotype (Table 1). To make a pure stock, the series of crosses shown in Figure 5 was performed. Five males were randomly selected for individual matings to the females of the *hk* as well as the balancer strains. Three of these males turned out to be Ddc^{ts2} */Ddc^{n7} heterozygotes. The other two were homozygotes and the one chosen for further work was designated as T10-1.

D. Identification of Canton-S transformants

The screen for Canton-S derived transformants was based on the assumption that a functional integrated *Ddc* gene would rescue the lethal phenotype of homozygous Ddc^{n7} mutant flies. All the surviving Canton-S G_0 flies (Table 2) were individually mated to the *n7/CyO* strain. The original second chromosomes were then replaced by those carrying Ddc^{n7} lethal mutant genes after two generations using the crossing scheme in Figure 6a. In this scheme, putative transformants were identified by their *rdo hk pr* phenotype in the G_2 generation. Table 4 shows the data of all such G_2 progeny from these crosses. All

Figure 5. Crossing scheme used to make a homozygous transformed line derived from injected *Ddc*^{ts2} embryos. Randomly selected T10-d males were individually mated to *hk* virgin females. The heterozygous males would yield *hk* as well as *non-hk* F_1 progeny, while the homozygous males would produce only *non-hk* progeny. Three days later these males were further mated to *n?/CyO* virgin females individually. Curly winged F_1 were selfed. The progeny of the homozygous males were saved for further study.


Ddc^ts2 * / n7 or
hk/hk X Ddc^ts2 * / Ddc^ts2 * X n7/CyO
(females) (males) (females)
Ddc^ts2 * / CyO
Only if no hk progeny appeared, would the crosses on the right be continued.
Ddc^ts2 * / Ddc^ts2 *

* indicates a chromosome carrying a newly acquired *Ddc* gene as a result of P element mediated transformation.

Figure 6a.

Crossing scheme used to replace *Ddc*$^{+}$ alleles by *Ddc*^{n7} alleles in the Canton-S derived G_0 adults. Canton-S G_0 flies were individually mated to the *n7/CyO* adults of the appropriate sex. The *Ddc*^{n7}/+ G_1 males (straight wings) were back-crossed to the balancer again. Among the G_2 progeny, those which showed *n7* homozygous phenotypes were saved.

6b.

Crossing scheme used to make homozygous transformed lines derived from the injected Canton-S embryos. The G_2 flies described in Figure 6a. were mated to the balancer stock. G_3 progeny was selfed. Among the G_4 progeny, those which showed the *non-Sb* phenotypes were considered as the homozygous transformants and saved for further study.

6a.

G_0 + / + ; * / + X *n?* / *CyO* ; + / +

v

G_1 *n?* / + ; * / + X *n?* / *CyO* ; + / +

or *n?* / + ; + / +

(males)

v

G_2 *n?* / *n?* ; * / +

n? / *n?* ; + / + (Die)

6b.

G_2 *n?* / *n?* ; * / + X *n?* / *CyO* ; *Sb* / *Ser*

v

G_3 *n?* / *n?* ; * / *Sb* X *n?* / *CyO* ; *Sb* / *Ser*

v

G_4 *n?* /*n?* ; * / *

* indicates a chromosome carrying a newly acquired *Ddc* gene as a result of P element mediated transformation.

TABLE 4. G_2 of Canton-S derived putative transformants

G_0 Adults †	Donor DNA Used	Sex	# *rdo*$^+$ *hk*$^+$ *pr*$^+$	# *rdo hk pr*	Stock Established
C10-1	p101ac	F	128	2	+
C10-3	p101ac	M	145	3	-
C10-13	p101ac	F	144	6	-
CZ-9	pZ	F	443	3	-
CZ-12	pZ	M	281	6	+
CZ-13	pZ	M	450	9	+
CZ-19	pZ	M	227	6	-
CZ-25	pZ	M	252	9	-

† Among all of the Canton-S derived G_0 adults, only these eight gave *rdo hk pr* progeny in the G_2 and were considered as putative transformants (Figure 7).

of the eight listed strains had at least two *rdo hk pr* G_2 progeny. This implies that all of them produced some homozygous Ddc^{n7}/Ddc^{n7} progeny, which survived due to the presence of an exogenous functioning *Ddc* allele (Ddc^{+} or Ddc^{+4}) in their genome. To establish homozygous stocks, the mating scheme listed in Figure 6b was carried out. Unfortunately, and somewhat unexpectedly, all of the Ddc^{n7}/Ddc^{n7}; */* G_4 flies were sterile. As a result, five of the putative transformants were lost. Only C10-1, CZ-12, and CZ-13 were conserved by promptly back-crossing heterozygous G_3 progeny to the balancer stock. After back-crossing for two generations, C10-1 and CZ-13 could be maintained in homozygous condition, (that is, Ddc^{n7}/Ddc^{n7}; */*), although still with lower reproduction than the wild type strain. However, CZ-12 could only be carried in heterozygous condition, which is Ddc^{n7}/Ddc^{n7}; */Sb.

E. Southern analysis of the transformants

The copy numbers of the inserted transposons in the transformed lines T10-1, CZ-12 and CZ-13 were examined by genomic Southern hybridization. High molecular weight DNA was prepared from small numbers of flies of these three transformed strains as well as the Canton-S control strain. The DNA was digested with Eco RI, then fractionated on a 0.7% agarose gel, transferred to a nitrocellulose filter and hybridized with a ^{32}P dCTP labeled 1.4 kb Hind III fragment (probe 3, see Figure 1). Figure 7 presents the autoradiograph of the Southern blot. DNA samples from the transformants all show a unique 6.6 kb fragment similar if not identical to that shown by the Canton-S sample. These bands represent the *Ddc* genes at their original location at 37C1-2 on chromosome 2L (Wright *et al.*, 1976; Hirsh and Davidson,

1981). Each transformant also reveals one additional fragment with a different length, 7.0 kb, 6.1 kb and 3.5 kb for the DNA samples of the T10-1, CZ-12 and CZ-13 lines, respectively. These bands represent the integrated genes at their new locations in the transformed lines. The different lengths of these fragments reflect the different distances from the transposon upstream to the nearest Eco RI sites. Since each tested strain shows only one additional fragment, the possibility that more than one integration event might have happened or that multiple inserts at one locus might have occurred is very unlikely.

F. In situ analysis of the transformants

Lines CZ-12, CZ-13 and T10-1 were further analyzed by cytological study. Late third instar larvae from these lines were collected. The salivary gland polytene chromosomes were prepared and hybridized in situ to a ^{3}H dTTP labeled nick translated 7.6 kb Pst I restriction fragment (probe 2, Figure 1), which was purified from p10lac plasmid DNA. Figure 8 presents sample autoradiographs. They show an extra hybridization site other than the band 37C1-2 on the second chromosome, the natural location of the *Ddc* gene. The additional site of T10-1 is at the band 21B, near the tip of the left arm of the second chromosome, while that of CZ-12 is at the band 99B, the right arm of the third chromosome.

The preliminary autoradiographs of CZ-13 showed that the additional *Ddc* site was on the multiply marked second chromosome, which had replaced the original second chromosome (Figure 6a). We do not have satisfactory explanation for this unexpected result, although it could be the result of a male recombination event which occurred during the

Figure 7. Southern analysis of the Canton-S and the transformed lines T10-1, pZ-12 and pZ-13. High molecular weight DNA was isolated from small numbers of flies according to Coen *et al.*, (1982). The DNA was restricted with EcoRI,and electrophoresed on a 0.7% agarose gel, then blotted onto a nitrocellulose filter. The DNA on the filter was hybridized with the oligo labelled probe 3 (Figure 1) and autoradiographed. Slots 1, 2, 3 and 4 contain the Canton-S, T10-1, pZ-12 and pZ-13 DNA, respectively.

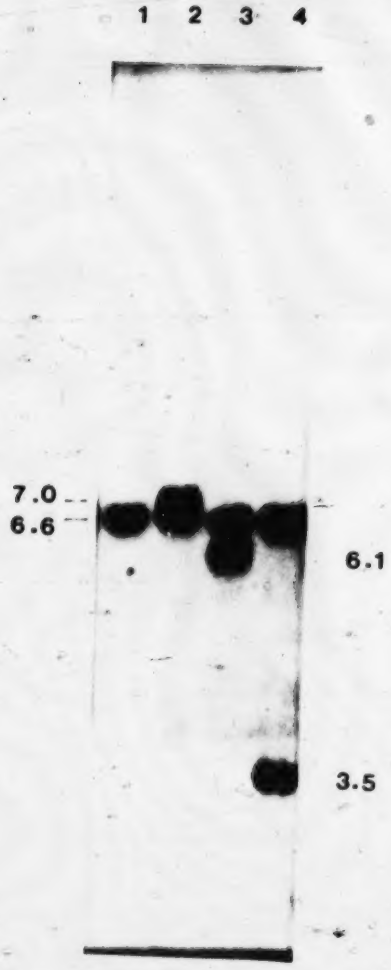

1 2 3 4
7.0
6.6
6.1
3.5

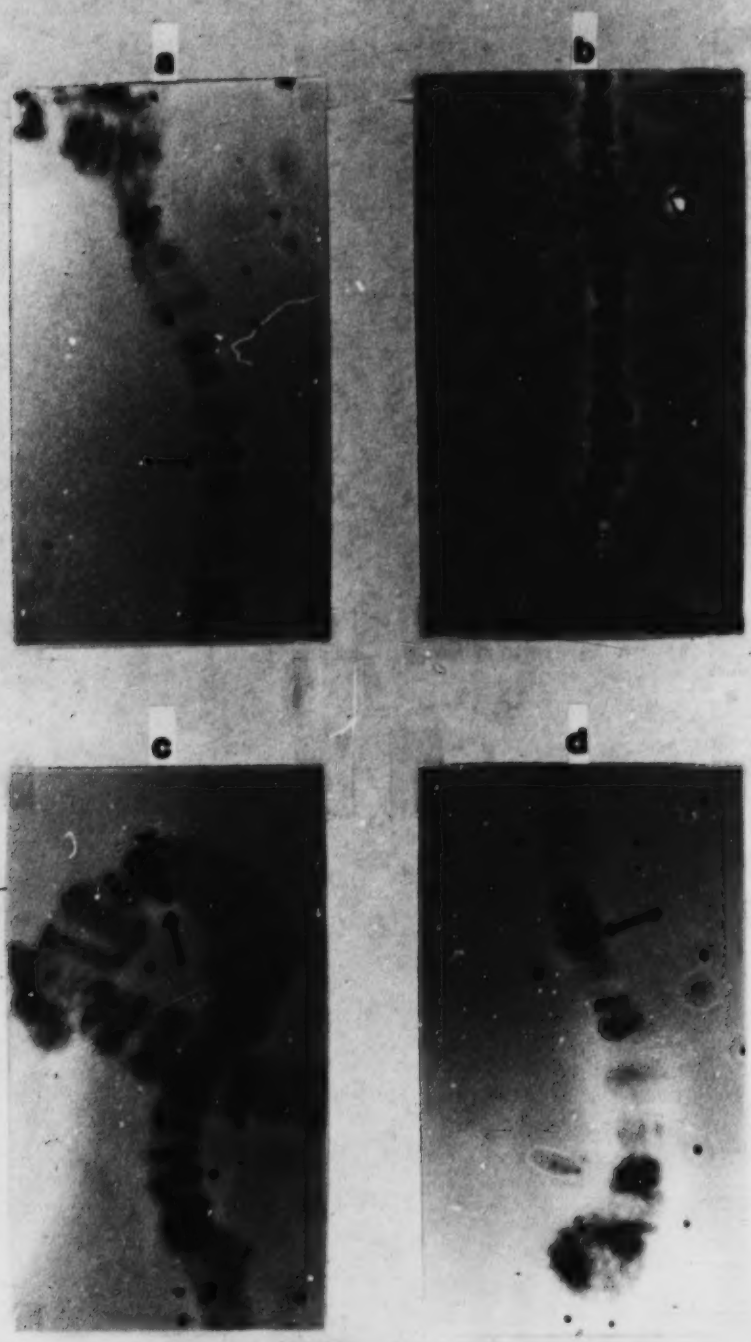
Figure 8. *In situ* analysis of T10-1, CZ-12 and CZ-13. *In situ* hybridization, autoradiography and photography were performed as given in the Materials and Methods. Preparations were probed with nick translated probe 2 (Figure 1). Arrowheads indicate areas of hybridization.

a. hybridyzation over band 37C1-2 on chromosome 2L, the original location of *Ddc* allele.

b. hybridization over band 21B on chromosome 2L, the exogenous *Ddc* gene location in line T10-1.

c. hybridization over band 99B on chromosome 3R, the exogenous *Ddc* gene location in line CZ-12.

d. hybridization over band 53C on chromosome 2R, the exogenous *Ddc* gene location in line CZ-13.


a
b
c
d

course of selection. Further investigation of this possibility is being undertaken.

G. DDC activity and protein assays

To test the proper regulation of the integrated transposons, the DDC specific activity of the three transformed lines was monitored at pupariation and eclosion. Non-transformed Canton-S wild type and the variant *Ddc*$^{+4}$ were used as the control strains. The animals at the proper developmental stages were collected. The DDC activity and the total protein content were measured. The specific enzyme activity was calculated in units per mg total protein weight. The data are presented in Table 5. The ratio of the activities at pupariation and eclosion was obtained. This ratio can be used to differentiate the wild type from the *Ddc*$^{+4}$ variant (see discussion for detail). The line T10-1 carries two transduced wild type *Ddc* copies and two *Ddc*^{ts2} copies. The *Ddc*^{ts2} alleles only contribute about 1% of the enzyme activity of the wild type strain (Wright *et al.*, 1982). However, the transformant showed only about half as much DDC activity as the wild type control did. We believe it may be caused by a chromosomal effect, which will be discussed later. The line CZ-12 has only one copy of the P[Ddc^{+4}] transposon while the line CZ-13 has two in its genome. In addition, both of them have two *Ddc*^{n7} mutant alleles which behave as null alleles (Clark *et al.*, 1978). The measurements taken from these two lines show an activity profile similar to *Ddc*$^{+4}$ which is characterized by significantly lower activity at pupariation than eclosion. As expected, the heterozygous line CZ-12 had about 50% DDC activity of the variant control strain reflecting the strict proportionality between gene

Table 5. DDC activity during development of Canton-S, *Ddc*$^{+4}$, T10 1, CZ-12 and CZ-13 strains

Strains	DDC activity (units/mg protein)†		Ratio of
	Pupariation	Eclosion	Pupariation/Eclosion
Canton-S	392 ± 21	280 ± 17	1.40
Ddc^{+4}	222 ± 13	381 ± 11	0.58
T10-1	196 ± 13	109 ± 18	1.80
CZ-12	107 ± 12	173 ± 14	0.62
CZ-13	249 ± 9	369 ± 41	0.67

†. Mean of 6 determinations on two collections ± standard deviation.

dosage and enzyme levels first noted by Hodgetts (1975). These observations suggest that all the transformants have the same pattern of enzyme activity which characterized the strains from which their exogenous genes were originally derived.

DISCUSSION

A. Selection of the transformants

Selection of transformants among the offspring of injected flies is usually based on phenotypic changes induced by the new gene carried by the P element transposon, and special mutations such as *ry* or *Adh* are usually required in the host strain(Spradling and Rubin, 1982; Rubin and Spradling, 1982; Goldberg *et al.*, 1983; Lis *et al.*, 1983; Dudler and Travers, 1984; DeCicco and Spradling,1984; Zehring *et al.*, 1984; Cohen and Meselson, 1985; Garabedian *et al.*, 1985, and Hiromi *et al.*, 1985; Hultmark *et al.*, 1986).

Recently, a neomycin resistance gene *(neoR)* has been inserted into a P element vector and used as a selective marker (Steller and Pirrotta, 1985). The functioning of this bacterial gene rendered the transformed *Drosophila* larvae resistant to the antibiotic G418 when present in the food at a concentration of 1 mg/ml, which is otherwise toxic to the animals. Since this transposon marker is selectable in any genetic background, no special mutant strains are required as the recipients. However, the transformants which express the *neoR* gene at a low level may be missed by the screen because of the chemical selection.

In previous transformation studies of *Ddc* using P element transposons, the temperature sensitive mutant strain, Ddc^{ts2}, was used as the host strain (Scholnick *et al.*, 1983; Marsh *et al.*, 1985). Ddc^{ts2} pupae develop a yellow-green colour at 25°C and "escaper" phenotype (a darkening at the extremes of the pupal case and an overall washed out colour) at 29°C in contrast to the reddish-brown wild type

pupae. Selection of the transformants was thus based on the phenotypic change of the *Ddc*^{ts2} mutant host strain to the wild type.

Unfortunately, in our hands, the *Ddc*^{ts2} strain was a very poor host in the transformation experiments. We showed that the embryos derived from *Ddc*^{ts2} strain were not as hardy as those from the Canton-S wild type. As mentioned in the Results, 77% of the Canton-S embryos hatched after being micro-manipulated (except injection), while only 33% of the *Ddc*^{ts2} embryos hatched following the same manipulation. In addition, approximately 22% of the Canton-S derived first instar larvae survived the injection and managed to eclose, while only 12% of the injected *Ddc*^{ts2} larvae developed into adulthood as indicated in Table 2. These results prompted our use of the wild type strain as the most appropriate host for P element mediated germ line transformation.

We did obtain one transformant T10-1, using the *Ddc*^{ts2} host, in which a copy of the *Ddc* gene present in Canton-S was integrated near the tip of 2L. The remaining three however were recovered following the injection of Canton-S hosts. This required replacement of the wild type alleles at the *Ddc* locus by mutant alleles following the integration event (see Figure 6a).

This selection system is very sensitive. Theoretically the minimum expression of the integrated transposon, consistent with survival, could be recovered. In our case, less than 1% of the wild type DDC activity in a viable transformed line is sufficient for life (Wright *et al.*, 1982), and presumably transformants with DDC levels this low could be recovered. However, none of the transformants we recovered had such low levels of DDC activity (Table 5).

The major problem of the protocol we ultimately used is that a

considerable proportion of the transformants (about 2/5) could not be recovered. If the exogenous DNA happened to integrate into the chromosomes to be replaced (the second chromosomes in this study), the selection scheme results in their being discarded (see Figure 6a). Had we chosen a heterozygous strain *(n7/CyO,* for example) as host, half of the previously discarded potential transformants might have been recovered, since exogenous *Ddc* gene integration on the *n7* chromosome could have occurred. Furthermore, if a heterozygous strain had been used as the recipient, it would have taken only one generation to replace the original wild type allele. However, one should be aware that a quarter of the embryos (those that are *CyO/CyO)* from such strains are lethal and will not be rescued. We did not use a heterozygous host since the recovery of every possible transformants was not our essential interest. It should be noted though, that the genetic scheme we have employed is generally applicable in P element mediated transformation. When there is no conditional mutation available, or when the exogenous gene product is indistinguishible from the original one, the replacement of the original wild type allele is almost unavoidable.

B. Expression of the P[Ddc$^+$] transposon

It has been demonstrated that a P[Ddc$^+$] transposon carrying the 7.6 Kb Pst I fragment of *Ddc* gene region contains all the essential information for tissue and temporally specific expression of the *Ddc* gene (Scholnick *et al.*, 1983). Marsh *et al.*, (1985) reported that the expression of the integrated transposons at different chromosomal locations varied within about 30% of the wild type controls. In this

study, the transformed line T10-1 carries the P[Ddc$^+$] transposon at a single integration site within its genome as demonstrated by both Southern analysis (Figure 7) and cytological mapping (Figure 8). However, it exhibits about 50% overall underproduction of the wild type controls as shown in Table 5.

In the previous studies on *Ddc* mentioned above, all the enzyme activity measurements were made in strains carrying two copies of the *Ddc^{ts2}* gene in addition to the introduced genes. In T10-1, the flies were also carrying two *Ddc^{ts2}* gene copies besides the integrated *Ddc$^+$* genes (Figure 5). The wild type *Ddc* region inserted in the transposon is the same length as that previously used by other groups (Scholnick *et al.*, 1983; Marsh *et al.*, 1985). Thus the chromosomal locations of these transposons would appear to be the only variable between the line T10-1 and the other reported transformants of *Ddc* . *In situ* hybridization of a *Ddc* probe to the line T10-1 polytene chromosomes showed that the exogenous *Ddc* gene was inserted near the tip of the left arm of the second chromosome (Figure 8). Could this integration site account for the lower expression of the transposon in the line T10-1 ?

There have been several cases in which exceptional, low levels of expression of the inserted transposons have been observed. Spradling and Rubin (1983) studied thirty six independently transformed lines bearing P[rosy$^+$]. Three of these lines had greater than a 50% reduction in the expression of the exogenous *rosy$^+$* gene. The lowest XDH specific activity measurement was reported as only about 30% of the wild type activity. Hazelrigg *et al.*, (1984) recovered two *white$^+$* transformants, which showed dramatically altered expression of the inserted gene. This

was revealed by a great reduction in the eye pigments and high sensitivity to repression by *zeste*^{1}. Normally, expression of the *zeste*^{1} phenotype requires the presence of two wild type copies of the *white* locus. However the presence of *zeste* in the transformants produced white eyed flies, consistent with a very low level of expression of the exogenous *white*$^{+}$ genes. Interestingly, in these two lines the P[white^{+}] transposon integrated near the centromere and the tip of the third chromosome, respectively. Chromosomal telomeres share with the centromere the characteristic of being heterochromatic in morphology. It has been revealed that there is DNA sequence homology between the repetitious DNA at the tips of all the *Drosophila* chromosomes and sequences within or near the centromeric heterochromotin (Young *et al.*, 1983). It is possible that the heterochromatic location of a transposon could greatly reduce the activity of an inserted gene, since position-effect variegation is ascribed to just such a phenomenon(Spofford, 1976).

Despite the low expression level, the data in Table 5 show that the ratio of the activities at pupariation and eclosion for T10-1 is greater than one which is characteristic of the Canton-S strain, from which the transposon DNA was derived.

C. Expression of P[Ddc^{+4}] transposons

Ddc^{+4} is a regulatory variant strain, which is phenotypically distinguishable from the Canton-S strain only by stage specific differences in DDC activity (Estelle and Hodgetts, 1984a). The *Ddc*$^{+4}$ gene does not produce any visible phenotype. The characterization of the transformants carrying a P[Ddc^{+4}] transposon therefore was based on

enzymatic measurements. Previous studies revealed that DDC activity measured in transformed lines bearing P[Ddc^{+}] transposons ranged within 30-35% of the wild type controls (Scholnick *et al.*, 1983; Marsh *et al.*, 1985). This range of variation was a concern to us, since *Ddc^{+4}* did not differ from *Ddc^{+}* levels by more than this. Thus enzymatic measurements from different lines at the same developmental stages would not likely yield conclusive data, since the variation inherent in the P[Ddc^{+4}] transposon would be indistinguishable from that caused by transformation *per se* (different integration sites, for example).

However, rather than comparing *Ddc^{+}* and *Ddc^{+4}* transformants at the same stage, we chose to compare within strain *ratios* of DDC activity at two developmental stages. The stages chosen were pupariation, where *Ddc^{+4}* shows an underproducer phenotype, and eclosion, where it shows an overproducer phenotype (Estelle and Hodgetts, 1984a). Measurements of the ratio of DDC activity at pupariation to that at eclosion (RPE) in *Ddc^{+4}* yielded a value of about 0.5, while the RPE from the wild type was about 1.4 (Table 5). When the RPE was re-calculated from the data of previous germ line transformation experiments by Marsh *et al.*, (1985), it was found that all of the transformed lines had a RPE of greater than one, including those that had multiple insertions within their genome (Table 6). This coincides with their conclusion that the P[Ddc^{+}] transposons were regulated correctly when integrated at a variety of chromosomal locations. The RPE of our transformed line T10-1 also showed a RPE of 1.8 (Table 5), similar to the wild type control, although its absolute DDC activity was much lower. These results support our belief that a RPE of greater than one characterizes the wild type, while a RPE of far less than one represents the *Ddc^{+4}*

Table 6

Relative DDC activity during development of the reported homozygous *Ddc* inserts †

Strains	DDC activity (uints/mg protein) Pupariation	Eclosion	Ratio of Pupariation/Eclosion
Canton-S	100	70.6	1.42
13A	130	62.5	2.08
24A	87.5	58.8	1.49
29A	77.5	56.3	1.38
30A	85	54.4	1.56
15C ¶	220	60.6	3.63
15F	162.5	79.4	2.05
19C *	322.5	248.8	1.30

†. The data in this table was re-calculated from Marsh *et al.*, (1985).

¶. This line has two independent *Ddc* inserts.

*. This line has three independent *Ddc* inserts.

phenotype. Therefore, the RPE of about 0.6 observed in the transformed lines CZ-12 and CZ-13 (Table 5) is typical of the *Ddc*$^{+4}$ strain, from which the inserted gene was originally derived. It is currently accepted that the chromosomal position affects the integrated gene expression in a more or less uniform manner at all developmental stages. Altered temporal expression patterns have been observed only when the associated regulatory sequences were altered (Pirrotta *et al.*, 1985; Levis *et al.*, 1985). We propose that the similarity of the temporal pattern of DDC activity in the lines CZ-12 and CZ-13 to that in the *Ddc*$^{+4}$ strain is due to the introduced P[Ddc^{+4}] transposon *per se*, and that the causative element(s) for the variant *Ddc*$^{+4}$ phenotype has been included in the 7.6 kb Pst I DNA fragment carried by the P[Ddc^{+4}] transposon.

Recently, DNA sequencing analysis of the 5' flanking region of the *Ddc*$^{+4}$ gene (Spencer, personal communication) has permitted a comparison to the corresponding sequence from Canton-S (Marsh and Hodgetts, personal communication). *Ddc*$^{+4}$ has a duplication of a 12 base pair AT-rich region about 280 base pairs upstream of the larval transcription start as positioned by Scholnick *et al.*, (1983). Although its functional significance is unknown, it is interesting to note that between -135 and -247 base pairs upstream of the ovalbumin gene there exists an 18 base pair AT-rich sequence, to which the progesterone receptor preferentially binds (Compton *et al.*, 1983). Secondly, the sequences required for the ecdysterone regulated expression of the hsp 23 gene are located more than 147 base pairs upstream of the capping site (Lawson *et al.*, 1985). It will be interesting to find out whether the altered DNA sequences of the *Ddc*$^{+4}$ mentioned above implies a

similar functional significance to these reported cases.

The molecular data also revealed that at about 20 base pairs upstream of the EcoR V site, located between the two EcoR I sites at 5' end (Figure 1), the variant has as an A to G transition, converting the enhancer-like 5'-ATGAAAA-3' sequence to 5'-ATGGAAA-3', which more precisely resembles the enhancer consensus sequence of mammalian viruses (Weiher *et al.*, 1983). It has been reported that an enhancer-like sequence lies upstream of the intermoult puff gene, *Sgs-4* which responds to regulation by 20-OH-ecdysone. A single base pair substitution adjacent to the putative enhancer at position -344 in a naturally-occurring variant of the *Sgs-4* gene is correlated with a 50% reduction in *Sgs-4* RNA (McGinnis *et al.*, 1983). Also a significantly increased level of gene expression was observed when the enhancer consensus sequences were fused to the 5' end of an *Adh* structural gene (Beckendorf, unpublished). Thus we speculate that this single base pair change in the Ddc^{+4} is responsible for the elevated level of the DDC activity seen in the variant.

If our hypothesis is right, then this effect was missed by Hirsh *et al.*, (unpublished) who indicated that only sequences downstream of the EcoR V site are required for the normal regulation of the *Ddc* gene. Because the 5' upstream regions of eukaryote genes may consist of multiple elements, each contributing a cumulative effect to the gene expression, the effect of changes in one or a few such elements could be easily missed. This is particularly true given the wide range of the variation of the DDC activity showed by the *Ddc* transformants, 30-35% in the reported cases (Scholnick *et al.*, 1983; Marsh *et al.*, 1985) and 50% in one of our inserts.

The major result of our study has been to show that the 7.6 kb Pst I fragment carries fundamental information for the *Ddc*$^{+4}$ variant phenotype. However, we have not proven that the element responsible for the overproducer phenotype has been included. Currently, functional tests of the Canton-S and *Ddc*$^{+4}$ enhancer-like sequences in the *Ddc* region, mentioned above, are being undertaken in our laboratory (C. Spencer, unpublished). A 40 bp synthetic oligomer containing these sequences has been fused onto the *Adh* structural gene, and the constructs are to be introduced into the *Drosophila* germ line. These experiments will test directly whether these sequences really function as an enhancer and may allow us to assign the overproducer phenotype of *Ddc*$^{+4}$ to this element.

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